Eco. Env. & Cons. 30 (February Suppl. Issue) : 2024; pp. (S292-S295)

ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2024.v30i02s.060

Biosynthesis of Bacitracin in Solid-state Fermentation using Different Substrates

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(Received 14 July, 2023; Accepted 11 September, 2023)

ABSTRACT

A *Bacillus* isolate was obtained from soil sample. It was then studied for its cultural, morphological and biochemical characteristics. The isolate was tentatively identified as *Bacillus subtilis*. For solid-state fermentation of bacitracin, the isolate was grown on basal medium. Fermentation was carried out SSF using three different solid substrates, wheat bran, rice husk and defatted cotton seed oil cake. Bacitracin produced was detected by TLC with standard bacitracin. The product formed in wheat bran showed greater inhibitory activity against test organism i.e. *Micrococcus luteus* with 20mm diameter of zone of inhibition.

Key words: *Bacitracin, solid-state fermentation, TLC, Wheat bran, Rice husk, Defatted cotton seed oil cake.*

Introduction

Fermentation is a process of breakdown of organic substances by microorganisms resulting in release of heat and the desired products (Hyseni *et al*., 2018). This is certainly an important process for several final products in food industry, but also for the production of several intermediate products. There are two main types of fermentations considering the free water content, Submerged Fermentation and Solid state fermentation. Solid state fermentation is the microbial cultivation process in the absence or near absence of free water in the substrate. However, enough moisture must be present in the substrate to support cell growth (Kapilan, 2015). Bacteria and filamentous fungi grow in symbiotic associations on solid substrates such as wood, seeds, stems, roots and leaves of plants. An antibiotic is a chemical substance used to kill or prevent growth of any other type of microorganisms at a lower dosage. Bacitracin is derived from cultures of *Bacillus subtilis*. It is a mixture of several polypeptides with different amino acid compositions (Ohki, 2003) and affects protein synthesis, cell wall synthesis and membrane functions. It is a potent antibiotic directed primarily against Gram positive organisms as well as some Archaebacteria (Arky, 1997). It is one of the important antibiotics used in human medicine and also used in animal husbandry for the prevention and control of diseases existing in farm animals (Rukmini *et al.*, 2015). Different types of bacitracin like A, A1, B, C, D, E, F, F1, F2, F3 and G have been discovered. The most potent antibiotic is Bacitracin A, most effective against Gram positive (+ve) and a few Gram negative (-ve) species of bacteria (Gulhane *et al.*, 2014).

Materials and Methods

Collection of soil samples

The soil sample was collected from a garden in Krishna Vishwa Vidyalaya (Deemed to be University), Karad. The sample was collected in a clean

polythene bag and was immediately bought to the laboratory.

Isolation of *Bacillus* species

1 g of soil sample was added in a tube containing 9 ml distilled water. This tube was kept in boiling water bath for about 15 min. the tube was cooled and serially diluted upto 10^{-6}. 0.1 ml of aliquote from each dilution was spread separately on sterile nutrient agar plate. These plates were kept for incubation at room temperature for 24 hr. The *Bacillus* being spore former sustains boiling temperature.

After incubation, the plates were observed for typical *Bacillus* colony with whitish color, irregular margin and flat elevation. A single colony with peculiar character was picked up and re-streaked on sterile nutrient agar plate for purification. The plate was incubated at room temperature for 24 hr. After purification, the culture was maintained on sterile nutrient agar slant and designated as BC1.

Characterization of the isolate

Presence of certain enzyme characteristics of genera or species of bacteria and test the ability of unknown organism to metabolize some of the common carbohydrates such as glucose, sucrose, lactose, maltose and mannitol were determined. Also, power to utilize hydrogen acceptors like sodium nitrate and sodium nitrite was studied. The spore staining was performed by Derner's method. The identification of isolate was done with reference to Bergy's manual of Systematic Bacteriology.

Production of bacitracin

Culture

A pure culture of an isolate BC1 was used for the production of antibiotic bacitracin. The culture was maintained on tryptone – glucose – YE agar medium.

Inoculum preparation

The bacterial growth was aseptically scrapped from 48 hrs old slants and transferred to 50 ml sterilized basal medium in 250 ml conical flask. Then it was kept on rotary shaker at 150 rpm for 24 hr at 30 °C. The vegetative culture thus obtained was used for inoculation into fermentation medium.

Media preparation

Three different substrates, viz. wheat bran, rice husk and defatted cotton seed cake were used for media preparation. The three flasks were separately added with the three substrates. Other ingredients were also added to these flasks. The pH was adjusted to 7. The flasks were sterilized at 121°C for 15 min at 15 lb pressure. After sterilization, the moisture was adjusted to 70% by adding sterile distilled water aseptically.

Fermentation

Each flask was inoculated with 2 ml of inoculum culture. The flasks were shaken well and kept for incubation for 48 hr at room temperature with intermittent shaking after 24 hr.

Extraction of crude bacitracin

After incubation period, the fermented material was soaked in N/100 HCl for 1 hr and filtered. The filtrate was centrifuged to remove residues at 4000 rpm for 15 min. the supernatant was used agar diffusion assay after neutralization using /100 NaOH. Detection of bacitracin by TLC

Preparation of TLC

The glass plates were used for TLC. The slurry of 10 g silica in 10 mL distilled water was prepared and coated on the glass plates uniformly. The plates were kept in oven at 160°C overnight for activation of gel.

Running sample

The three separate spots from three different samples (from the three fermentation flasks) were placed 2.5 cm from the bottom edge and equidistant from each other on the plate. A 10 µL/ml of standard bacitracin was also spotted at one end of TLC plate, parallel to the sample spots.

All the spots were dried for about 1 to 2 min. The plate was then kept in a chromatographic chamber containing equilibrated solvent system. The solvent system used contained n-Butanol: Acetic acid : Water in 4 : 2 : 1 ratio. As soon as the solvent reached the top of the plate, dry TLC plate was sprayed with iodine solution and the distance travelled by each spot was measured.

Bacitracin assay

The activity of bacitracin present in the fermented material was determined by agar diffusion method. The nutrient agar was used for bioassay. On asterile nutrient agar plate, the test organism (*Micrococcus*

luteus) was spread uniformly. A well was prepared at the centre of the plate. The sample was added in the well with the help of a sterile syringe. The plates were kept at 4°C for 20 min for diffusion of content in well. Then the plates were incubated at 37°C for 24 hrs.

Results and Discussion

Isolation of *Bacillus* species

A single isolate of *Bacillus* species was isolated on sterile nutrient agar plate. It was designated as BC1. The cultural characteristics of the isolate were recorded and are listed in Table 1 and 2. The isolate was found to be Gram positive short rod. It was highly motile with centrally placed endospore. From Table 3, it is clear that the isolate gave positive tests for citrate utilization, Voges – Proskauer, oxidase, catalase, starch hydrolysis, nitrate reduction, casein hydrolysis.

Detection of bacitracin

The detection of bacitracin production was comparable with that of pure standard bacitracin, with R_f value of 0.34. Table 4 shows R_f values of sample spots obtained after TLC.

Bacitracin assay

The activity of bacitracin present in the fermented material was determined by agar diffusion method. After incubation with test organism, the plates showed inhibition zones. The samples obtained from wheat bran, rice husk and defatted cotton seed cakes showed zone of inhibitions of diameters 20 mm, 16mm and 17 mm respectively, as shown in Photoplate 1.

Table 2. Morphological characteristics of BC1

Gram staining	Gram positive
Spore formation	Centrally localized endospore
Motility	Highly motile

Table 3. Biochemical characteristics of BC1

Sr. No.	Test	Result
1	Citrate utilization	+
2	Methyl red	-
3	Voges – Proskauer	+
4	Oxidase	+
5	Catalase	+
6	Starch hydrolysis	+
7	Nitrate reduction	+
8	Casein hydrolysis	+
9	Gas production from glucose	-
10	Gas production from sucrose	-

Table 4. R_f values obtained after running TLC

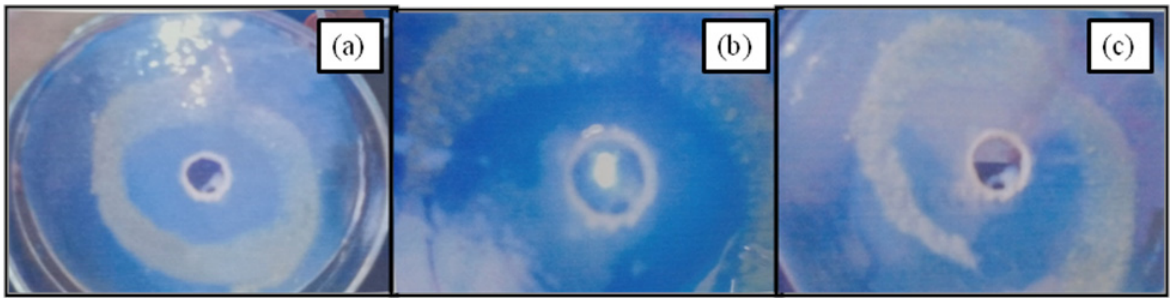
Sr. No.	Sample	R_f value
1	Standard bacitracin	0.34
2	Sample 1 (wheat bran)	0.32
3	Sample 2 (rice husk)	0.30
4	Sample 3 (defatted cotton oil seed cake)	0.33

Conclusion

The three solid substrates, viz. Wheat bran, rice husk

Table 1. Cultural characteristics of BC1

Size	Shape	Colour	Margin	Opacity	Surface	Elevation	Consistency
1 mm	Circular	White	Irregular	Opaque	Smooth	Raised	Moist



Photoplate 1: Photographs showing formation of zones of inhibition by bacitracin for wheat bran, (b) rice husk and (c) defatted cotton seed cakes

and defatted cotton seed oil cake are proven to have ability to support growth of *B. subtilis*. They also promoted bacitarcin production in the same organism when grown for 48 hr. Wheat bran seems to be the best source of C and N than the rest of two substrates as it shows higher antibiotic activity. The solid state fermentation of bacitracin using crude substrate like wheat bran is cost effective for large scale production.

Conflict of interest

There is no any conflict of interest.

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